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Biosynthesis of the peroxisomal dihydroxyacetone synthase from *Hansenula polymorpha* in *Saccharomyces cerevisiae* induces growth but not proliferation of peroxisomes

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Summary. The *DAS* gene of *Hansenula polymorpha* was expressed in *Saccharomyces cerevisiae* under the control of different promoters. The heterologously synthesized dihydroxyacetone synthase (DHAS), a peroxisomal enzyme in *H. polymorpha*, shows enzymatic activity in baker's yeast. The enzyme was imported into the peroxisomes of *S. cerevisiae* not only under the appropriate physiological conditions for peroxisome proliferation (oleic acid media), but also in glucose-grown cells where it induced the enlargement of the few peroxisomes present. This growth process was not accompanied by an increase in the number of microbodies, which suggests a separate control mechanism for peroxisomal proliferation.

Key words: Peroxisomes – Protein import – *Saccharomyces cerevisiae* – *Hansenula polymorpha*

Introduction

In contrast to the information available on protein targeting into chloroplasts or mitochondria, little is known about the molecular mechanism of protein targeting into peroxisomes. Only recently have the first targeting signals for protein import into peroxisomes been described. In the peroxisomal acyl CoA oxidase of the yeast *Candida tropicalis*, two regions have been identified that mediate protein import (Small et al. 1988), and a short sequence of amino acids at the carboxy-terminus of insect luciferase has been shown to act as a peroxisomal targeting signal in mammalian cells (Gould et al. 1987). Methylotrophic yeast species promise to be good model organisms for studying peroxisomal biogenesis (Veenhuis et al. 1983), because the proliferation and the enzymatic composition of their peroxisomes can be strongly influenced by growth conditions. The best studied species of these yeasts are *Hansenula polymorpha, Candida boidinii* and *Pichia pastoris*. Growth on methanol as a sole carbon and energy source results in the appearance of many large peroxisomes, which can comprise up to 80% of the total cell volume. The first steps of methanol assimilation and dissimilation take place in these organelles (Veenhuis et al. 1983).

Recently, genes encoding some of the peroxisomal proteins have been cloned and sequenced (Ellis et al. 1985; Janowicz et al. 1985; Ledeboer et al. 1985). Sequence analyses have revealed that these enzymes, which are synthesized in the cytoplasm and assembled post-translationally into the peroxisomes (Goodman et al. 1984; Goodman 1985) have apparently no cleaveable signal sequence for translocation (Roa and Blobel 1983; Roggenkamp et al. 1984; Ledeboer et al. 1985). The development of transformation systems for *H. polymorpha* and *P. pastoris* provides a molecular approach to peroxisomal protein translocation analysis (Cregg et al. 1985; Roggenkamp et al. 1986).

In *Saccharomyces cerevisiae* the β-oxidation pathway is located in the peroxisomes. It has recently been shown that the proliferation of these organelles is inducible by growing *S. cerevisiae* on oleic acid (Veenhuis et al. 1987). This induction method now allows the analysis of peroxisomal biogenesis in an organism that is amenable to extensive genetic analysis.

In order to explore the use of heterologous peroxisomal proteins for the study of peroxisomal biogenesis, we have expressed the *H. polymorpha* genes *DAS* and *MOX* which encode, respectively, dihydroxyacetone synthase and methanol oxidase, in *S. cerevisiae*. As only the *DAS* gene was found to be expressed as an active enzyme, we consequently studied the subcellular localization of the dihydroxyacetone synthase protein in *S. cerevisiae*. The analysis of this heterologous

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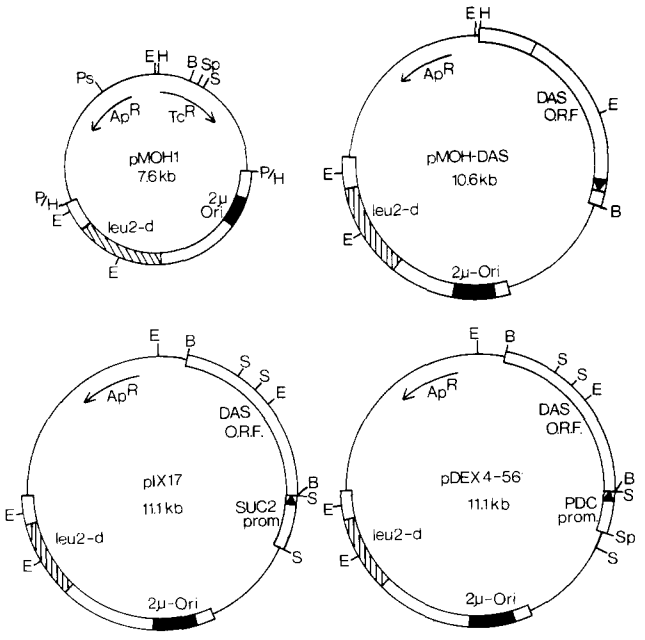


Fig. 1. The structures of the plasmids that were used to express the *DAS* gene in *S. cerevisiae*. Plasmid pDEX4-17 ist not shown because its overall structure does not differ from that of plasmid pDEX4-56. For details of construction, see Materials and methods. *B* BamHI, *E* EcoRI, *H* HindIII, *P* PvuII, *S* SalI *Sp* SphI, *P/H* fusion site of PvuII and HindIII, Ap^R and Tc^R ampicillin and tetracycline resistance gene, respectively

protein by cell fractionation and immunocytochemical methods revealed that *S. cerevisiae* is able to import the enzyme into its peroxisomes. We further demonstrated that the presence of DHAS in *S. cerevisiae* induces growth, but not proliferation of the few peroxisomes present in the cells during growth on glucose. During the course of this work peroxisomal import of inactively expressed methanol oxidase in *S. cerevisiae* was reported independently (Distel et al. 1987).

Materials and methods

Strains and culture conditions

S. cerevisiae strains EK1 (a *leu2-3, leu2-112, pep4-3*) (Kellermann et al. 1986) and PS1 (a *leu2-3, leu2-112, ura3-52, his4-519, pep4-3*; P. Seeboth, this institute) were used for transformation with recombinant plasmids. Cultures were grown on 0.67% YNB, 3% glucose supplemented with amino acids (40 μg/ml) or uracil (20 μg/ml). Under derepressing conditions cultures were grown on 0.67% YNB, 3% lactate supplemented with amino acids and uracil if necessary. For induction of peroxisomes, cultures were grown on YNB, glucose until mid-log phase and were then transferred to 0.1% yeast extract, 0.1% oleic acid and 0.05% Tween 20.

H. polymorpha (ATCC 34438) was grown on 0.67% YNB, 1% methanol (induced) or 0.67% YNB, 3% glucose (repressed conditions). All plasmid constructions were carried out in *E. coli* JA221 (*recA1, leuB6, trpE5, hsdR⁻, hsdM+, lacY*).

Plasmids

pMOH1 (Fig. 1), a yeast-*E. coli* shuttle vector, was constructed by cloning a blunt-ended 3.3 kb HindIII fragment from pMP78 (Hollenberg, 1979), which carries the *leu2-d* allele and the 2-μm DNA origin of replication, into the PvuII site of pBR322. The plasmid pMOH-DAS (Fig. 1) was obtained after ligation of HindIII-BamHI-digested pMOH1 with a 3.3 kb HindIII-BamHI fragment from plasmid YRp7-DAS (Janowicz et al. 1985), the latter containing the complete *DAS* ORF and 1 kb of the 5′ non-coding region of the *DAS* gene.

Plasmids pDEX4-17 and pDEX4-56 were constructed as follows. A SphI-BamHI fragment of the *PDC1* promoter, which ends at the 3′ end at position −112 relative to the translation initiation codon, was inserted into the SphI-BamHI digested pMOH1: the result is plasmid pPDC4. DNA fragments containing the coding region of the *DAS* gene were inserted, after Bal31 treatment and linker attachment, as BamHI fragments into the BamHI site of pPDC4. The deletion endpoints of the fragments used are located at positions −17 and −56, respectively, resulting in plasmids pDEX4-17 and pDEX4-56 (Fig. 1).

Plasmid pIX17 was obtained by replacing the 0.8 kb SalI fragment carrying the *PDC1* promoter fragment with a 0.8 kb *SUC2* promoter fragment from plasmid pRB58 (Carlson and Botstein, 1982). The original EcoRI-HindIII fragment was supplied with SalI linkers after endfilling the restriction sites. This fragment was then inserted into pDEX4-17 after partial digestion with SalI (Fig. 1).

Standard techniques were used for cloning and sequencing of DNA (Maniatis et al. 1982; Maxam and Gilbert 1980). Yeast transformations were carried out following a procedure described by Klebe et al. (1983). SDS polyacrylamide gel electrophoresis and protein transfer were carried out according to published procedures (Laemmli, 1970; Towbin et al. 1979). Dihydroxyacetone synthase (DHAS) was isolated from induced *H.polymorpha* as described (Bystrykh et al. 1981). Antibodies against the purified protein were raised in rabbits using standard procedures. DHAS was detected on Western blots by visualization of antibody binding after incubation of the blots with horseradish peroxidase protein A by oxidation of diaminobenzidin.

For determination of DHAS activity, transformants were grown to mid-log phase, harvested by centrifugation for 5 min at 6,000 *g* and washed in 20 m*M* sodium phosphate buffer, pH 7.5. Cells from 50-ml cultures were resuspended in 0.5 ml of the same buffer containing 0.1 m*M* PMSF, and then were broken with glass beads in a Braun homogenizer for 3 × 2 min at 4°C. Cellular debris was removed by a 5-min centrifugation in an Eppendorf centrifuge, and then aliquots of the crude extracts were passed through a DEAE sephacel column equilibrated with 20 m*M* sodium phosphate buffer,pH 7.5, to remove interfering enzymatic activities. Under these conditions DHAS does not bind to DEAE sephacel, but is found in the flow-through. The DHAS assay was performed as described by Bystrykh et al. (1981).

Standard procedures were followed for measuring catalase and cytochrome c oxidase (Roggenkamp et al. 1974; Tolbert 1974).

Cell fractionations were carried out essentially as described for *C. boidinii* (Goodman et al. 1984; Goodman 1985), except for the sucrose gradients. Continuous gradients of 30%–50% sucrose loaded onto a cushion of 60% sucrose were used. Fractions were harvested by collecting drops from the bottom of the gradients.

Preparations of cells for electron microscopy and immunocytochemical treatments were carried out as described by Douma et al. (1986), cytometric analysis as described by Veenhuis et al. (1978).

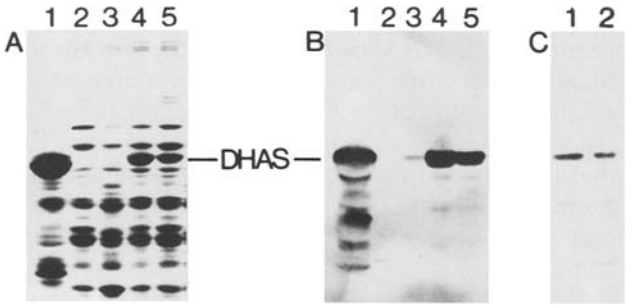


Fig. 2A–C. Analysis of protein extracts of *S. cerevisiae* EK1 transformants by SDS-PAGE and Western blotting. **A** SDS gel; **B** corresponding immunoblot of transformants containing plasmids pDEX4-56 (*3, 4*) and pDEX4-17 (*5*). Extracts of methanol-grown *H. polymorpha* (*1*) and untransformed *S. cerevisiae* EK1 (*2*) were added as controls. In lactate-grown transformants of pDEX4-56 (*3*) a drastical reduction in DHAS synthesis in comparison to glucose-grown cells (*4*) is observed. An extract of glucose-grown pDEX4-17 transformants also shows high-level expression of the *DAS* gene (*5*). **C** Western blot of *S. cerevisiae* EK1 transformants bearing plasmid pMOH-DAS grown on YNB, glucose (*1*) and YNB lactate (*2*)

Table 1. Specific activities of DHAS in different *S. cerevisiae* EK1 transformants grown on YNB, 3% glucose

Plasmid	Specific activity[a]
pMOH-DAS	0
pDEX4-17	0.014
pDEX4-56	0.019

[a] μmol formaldehyde converted/min per milligram protein

Results

Expression of the DAS gene in S. cerevisiae

To study the behaviour of the heterologous peroxisomal DHAS in *S. cerevisiae* we tested different expression plasmids carrying the *DAS* gene of *H. polymorpha* for their ability to express the gene in *S. cerevisiae* EK1. In a first construction, pMOH-DAS (Fig. 1), the *Hansenula* promoter directed transcription in *S. cerevisiae*. Although the gene was cloned into a high copy number 2-μm derived plasmid, its expression on YNB-2% glucose medium was very low. The protein could be detected only by immuno analysis on Western blots (Fig. 2C). To test the possibility of whether gene regulation was the same in *S. cerevisiae* as in *Hansenula*, i.e. by a glucose repression/derepression mechanism, protein extracts from cells grown on YNB-3% lactate were examined for the presence of the DHAS protein. On Western blots equal amounts of DHAS were detectable both in glucose- and lactate-grown cells (Fig. 2C). This suggests that the *Hansenula* promoter is only very poorly recognized by the transcription apparatus of *S. cerevisiae* and that the weak expression reflects a constitutive basal level of transcription of the gene. Under both conditions no DHAS activity could be detected.

In order to increase expression, the *DAS* ORF was placed under control of the *PDC1* promoter in plasmids pDEX4-17 and pDEX4-56 (Fig. 1). In crude extracts of the transformants of *S. cerevisiae* EK1 carrying these plasmids, a high level of expression was detected. Analysis of the protein patterns of the transformants on the Coomassie blue-stained gel revealed an additional band having the size of the DHAS polypeptide. This additional band was one of the most prominent protein bands present in the gel, suggesting that DHAS is produced in large amounts in these transformants (Fig. 2A). The immunoblot shows a strong antibody reaction at the position of this extra band, indicating that it does indeed represent the DHAS protein (Fig. 2B). The extract of untransformed cells showed no such signal.

Protein levels, as estimated by densitometric scanning of the stained gel strips, approximated 4% DHAS in pDEX4-17 transformants and 9% DHAS in pDEX4-56 transformants.

DHAS enzymatic activity was detected in the protein extracts of both transformants of pDEX4-17 and pDEX4-56 (Table 1).

We also investigated whether the expression of the *DAS* gene under control of the *PDC1* promoter is regulated. It was shown previously that this promoter is fully induced during growth on glucose media, but only a reduced expression level was observed on lactate media (Schmitt et al. 1983). A comparison of crude extracts of pDEX4-56 transformants showed a drastic decrease in the expression of the *DAS* gene in lactate-grown cells relative to glucose-grown cells (Fig. 2A, B). The expression of the *DAS* gene was also reduced on yeast extract/oleic acid media, a result of regulation of the *PDC1* promoter. The decrease in *DAS* transcription on this medium was detected by Northern analysis (data not shown).

Subcellular localization of DHAS in S. cerevisiae

In a first attempt at analysing the localization of the DHAS protein in *S. cerevisiae*, we analysed transformants of the plasmid pDEX4-56 by cell fractionation methods. The cells were first grown on YNB-3% glucose to mid-log phase and then shifted to oleic acid media. After a 13-h induction period on oleic acid medium the distribution of the DHAS protein between the cytoplasmic and the organelle fractions was analy-

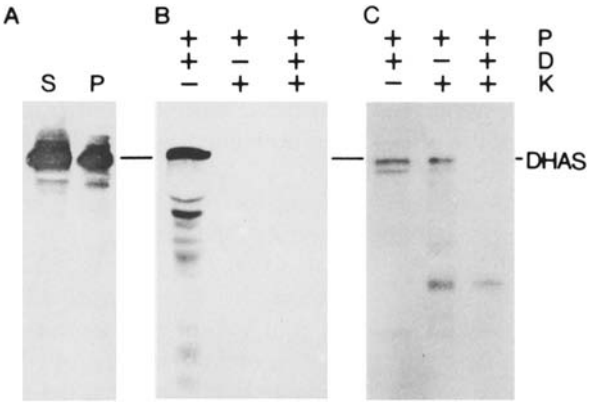


Fig. 3A–C. Analysis of the subcellular distribution of DHAS in pDEX4-56 transformants grown on oleate medium. **A** Distribution of DHAS between cytoplasmic and organelle fractions. Cells were fractionated into a cytoplasmic (*S*) and an organelle fraction (*P*). Equal volume fractions of both were analysed for DHAS content by SDS-PAGE and Western blotting. Under peroxisome-inducing conditions the S17 contained more than 50% of the DHAS present in the cells. **B** Protease treatment of cytoplasmic proteins (*S*) from oleate-induced pDEX4-56 transformants. Aliquots containing 30 μg of the *S* fraction were treated with proteinase K in both the presence and absence of detergents. The samples were incubated in 1 *M* Sorbit, 5 m*M* MES (pH 5.5) for 20 min at room temperature. If detergents were added, the final concentration was 1% Triton X-100 and 1% sodium desoxycholate; if proteinase K was added, the final concentration was 500 μg/ml. The total volume of the reaction mixtures was 200 μl. The reactions were stopped by the addition of PMSF to a final concentration of 2 m*M*, 50 μg BSA was then added, the samples were precipitated with acetone and finally analysed by SDS-PAGE and Western blotting. The composition of the reaction mixtures is listed above each lane. (*P* protein fraction, *D* detergent mix, *K* proteinase K). **C** Protease treatment of organelle fraction (*P*). Protocol as in **B**; 20 μg proteins were used in each lane

sed. As shown in Fig. 3A considerable amount of DHAS sedimented with the organelles when osmotic shock lysates obtained from protoplasts were centrifuged at 17,000 *g*. However, more than 50% of the DHAS protein was found in the soluble cytoplasmic S17 fraction (Fig. 3A). This suggests that at least some of the DHAS was particle-bound in oleate-grown *S. cerevisiae*.

In order to test whether the sedimentable DHAS indeed represented organelle-imported protein, the protease resistance of the DHAS in aliquots of the S17 and P17 fractions was investigated in the presence and absence of detergents (Fig. 3C). The results indicate that the DHAS in P17 was highly protected against protease digestion. The addition of detergent and protease led to the degradation of DHAS, while in samples containing only protease or only detergent DHAS remained detectable. In contrast to this result the DHAS in the S17 fraction was protease digestable even in the absence of detergents (Fig. 3B).

Several reasons can be given for the fact that only a portion of the DHAS was present in the peroxisomal fraction. In the first place peroxisomes are fragile and can be disrupted easily during the fractionation. Furthermore, it is possible that the molecules synthesized during the conditions of repression of microbody proliferation (glucose) resided in an import-incompetent state even after the shift of the cells to oleic acid media.

To obtain a high level of expression of DHAS concomitantly with the induction of peroxisomes on oleic acid medium, the *DAS* gene was placed under control of the *SUC2* promoter (pIX17). The *SUC2* promoter shows the highest activity under conditions of glucose derepression (Carlson and Botstein 1982). Plasmid pIX17 (Fig. 1), therefore, allows the simultaneous induction of peroxisomes and DHAS expression.

Transformants of plasmid pIX17 were analysed in a similar manner as described above. Thirteen hours after the cells had been transferred from glucose to oleic acid media a cell fractionation was performed (Fig. 4). Even under the conditions of simultaneous induction of peroxisome formation and *DAS* expression, 50% of the DHAS was not particle bound (Fig. 4A). After sucrose density gradient centrifugation most of the sedimentable enzyme co-migrated with the mitochondrial and peroxisomal fractions. Similar results were obtained with pDEX4-56 transformants (data not shown). In the case of oleate-grown cells, DHAS co-migrated with the peroxisomes and mitochondria, but surprisingly, we found the same distribution of DHAS in glucose-grown transformants. This suggests at least a partial import of DHAS under physiological conditions that normally repress microbody proliferation. Since our biochemical experiments did not allow an unambiguous localization of the DHAS protein under the different conditions described above, immunocytochemical means were employed.

Electron microscopy

The overall cell morphology of the transformants of plasmids pDEX4-56 and pIX17 is shown in Figs. 5–8. Figures 5 and 7 both represent glucose-grown cells, which normally contain only a few small peroxisomes. This is indeed the case in the transformant cells shown in Fig. 7 in which the biosynthesis of DHAS is repressed by the glucose present in the medium. When, however, DHAS was synthesized in such cells (Fig. 5), peroxisomes were larger and obtained the same size as in the cells grown under oleate-induced conditions (Figs. 6 and 8). This observation was supported by a morphometrical analysis (Table 2). The volume frac-

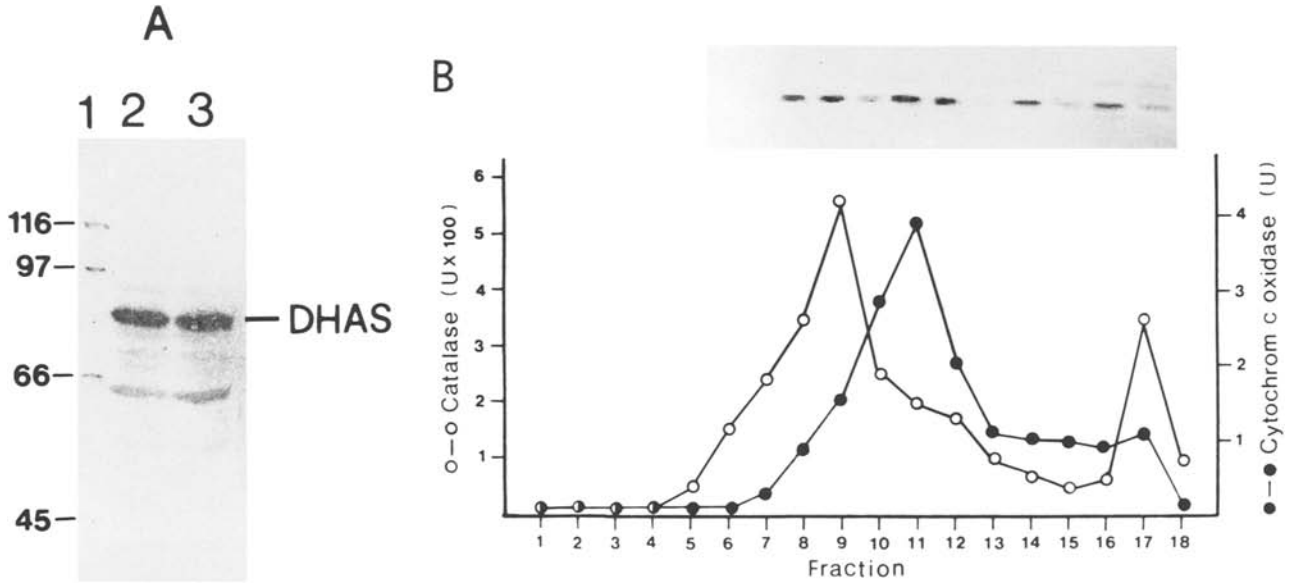


Fig. 4A, B. Analysis of the subcellular location of DHAS in pIX17 transformants. **A** Distribution of DHAS between cytoplasmic (*2*) and organelle fractions (*3*) after centrifugation at 17,000 *g*. Osmotic-shock lysates of protoplasts of pIX17 transformants grown for 13h on YNB oleic acid were analysed by immunoblotting for distribution of DHAS between supernatant and pellet after centrifugation at 17,000 *g*. Fifty percent of the DHAS was associated with the pellet (= organelle fraction). *1* MW marker: β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa). **B** Analysis of the organelle-associated DHAS by sucrose density gradient centrifugation. The P17 fraction was loaded on top of a 30%–50% sucrose gradient and spun for 6h at 27,000 *g* in a SW27 rotor. Fractions of 1.5 ml were then collected from the bottom of the tube, and 30 μl of each fraction was subsequently separated by SDS-PAGE and analysed by immunoblotting for the presence of DHAS. The resulting blot is aligned with the distribution of the marker enzyme cytochrome c oxidase (mitochondria) and catalase (peroxisomes). *1* bottom, *18* top

Table 2. Results of cytometric measurements of peroxisomes in *S. cerevisiae* transformants and control cells

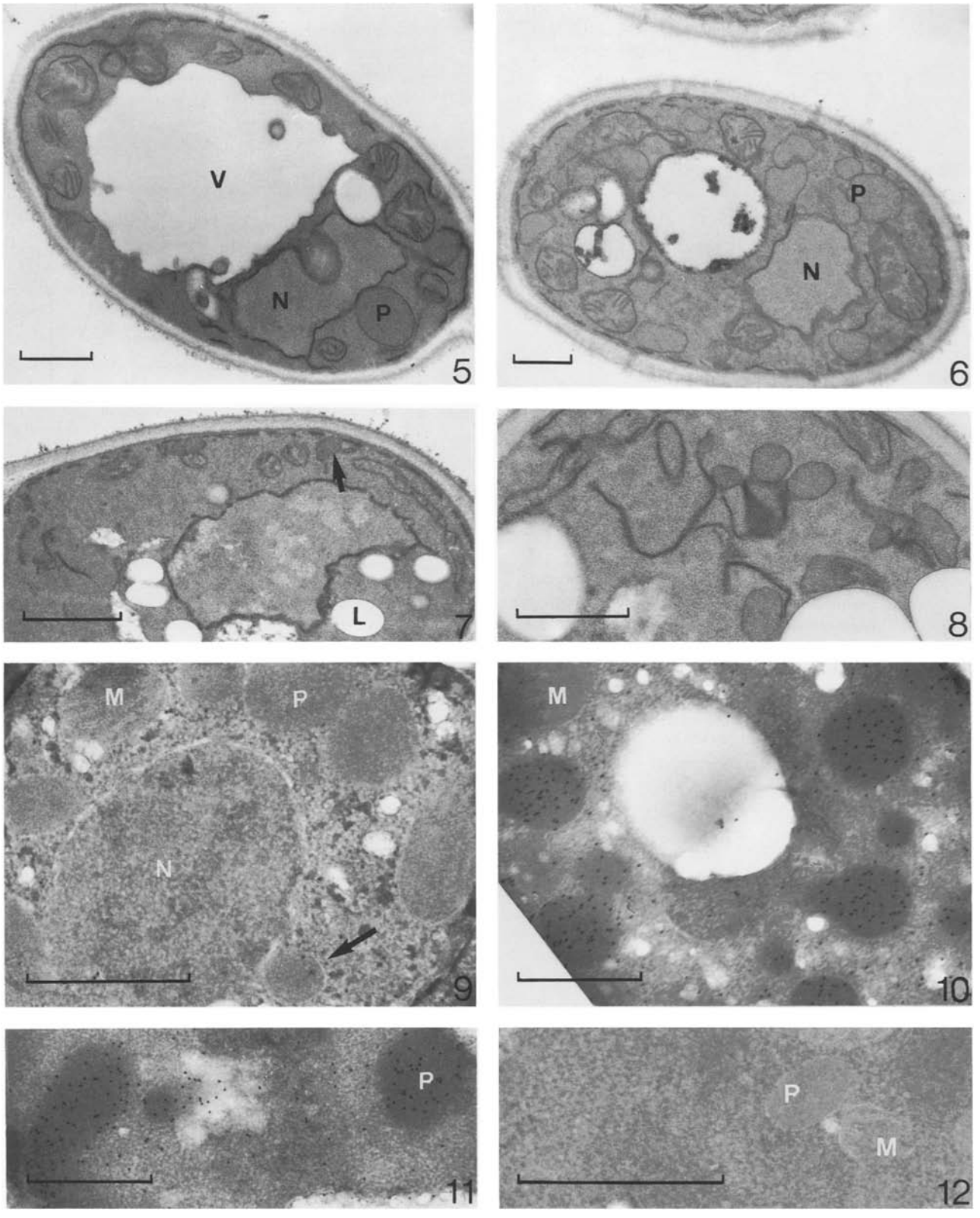
Carbon source	Strain	Number[a]	Volume fraction[b]
Glucose	n.t.[c]	n.d.[d]	0.2
	pDEX4-56	n.d.[d]	1.8
Oleic acid	n.t.[c]	2.6	6.4
	pIX17	2.7	11.1

[a] Average number per thin section (Veenhuis et al. 1978)
[b] Percentage of the cytoplasmic volume (Veenhuis et al. 1978)
[c] Non-transformed control cells
[d] Not determined

tions of peroxisomes are significantly increased in the transformants in comparison to untransformed control cells due to the high expression of the DHAS protein.

Serial sectioning of the glucose-grown cells indicated that the number of microbodies per cell did not increase in the DHAS-producing transformants (Fig. 5; pDEX4-56) compared to the pIX17 transformants (Fig. 7) in which the synthesis of DHAS was low or compared to wild-type cells (not shown). Generally, 2–5 microbodies were present in all strains: in pDEX4-56 transformants they attained a maximum diameter of 0.6 μm; in pIX17-harbouring cells and in wild-type cells, the maximum diameter of the organelles was 0.2 μm. This demonstrates that in glucose-grown cells the overexpression of a peroxisomal protein results only in an increase in peroxisomal size and not in the induction of peroxisome proliferation.

Proliferation occurred to the same extent in pIX17 transformants and wild-type cells on oleate medium. This is shown by a comparison of the average numbers of peroxisomes per thin section of pIX17 transformants and control cells (Table 2). The values 2.6 and 2.7 indicate that the number of peroxisomes is almost identical. It must be stressed that the values determined here are numbers of peroxisomes per section and, therefore, are not comparable to the number of peroxisomes determined by serial sectioning of glucose grown cells. These numbers only demonstrate that microbodies in the pIX17 transformants proliferate to the same extent as those in wild type cells grown on oleic acid. Under no conditions did we observe the proteinaceous aggregates described by Distel et al. (1987) in alcohol oxidase-producing strains of baker's yeast.



Figs. 5–8. Thin sections of $KMnO_4$-fixed cells showing the morphology of the two transformants grown on glucose (**Fig. 5**, pDEX4-56; **Fig. 7**, pIX17) and oleic acid media (**Fig. 6**, pIX17; **Fig. 8**, pDEX4-56). Note: when DHAS is highly expressed the size of the microbodies increases (**Figs. 5** and **6**). *L* lipoid droplet, *M* mitochondrion, *N* nucleus, *P* peroxisome. The marker represents 0.5 μm

The immunocytochemical results are shown in Figs. 9–12. Experiments performed on ultrathin lowicryl sections using specific antibodies against DHAS and protein A/gold revealed that under conditions of enhanced DHAS synthesis specific labelling is concentrated on the microbody profiles, although it is also present in the cytosol (Fig. 10; pIX17, oleic acid and Fig. 11; pDEX-56, glucose). When the same transformants were grown under conditions of low synthesis of DHAS, labelling was very weak (Fig. 9; pDEX4-56, oleic acid) or lacking completely (Fig. 12; pIX17, glucose).

Discussion

The *DAS* gene from *H. polymorpha* which encodes the peroxisomal dihydroxyacetone synthase, has been expressed in *S. cerevisiae* through the introduction of a variety of expression plasmids into this yeast. The genuine *H. polymorpha* promoter was recognized only very poorly by the *S. cerevisiae* transcription apparatus. This could be concluded from the fact that only small amounts of the enzyme were synthesized on both glucose media and lactate media. Fusions of the *DAS* coding region to *PDC1* or *SUC2* promoters led not only to an increased expression of the gene but also to its regulatable expression. High amounts of DHAS protein could be expressed on 3% glucose media in *S. cerevisiae* EK1 transformed with plasmid pDEX4-56 (8%–10% of total cell protein). Enzymatic activity could only be detected in the highly expressing transformants of the PDC1-DAS fusions. The specific activity of the enzyme, however, lies below the values expected for a completely active gene product. On the basis of a specific activity of 4 U/mg (as determined for the purified enzyme, Bystrykh et al. 1981), one would expect specific activities of 0.3–0.4 U/mg in crude extracts of pDEX4-56 and activities of 0.1–0.2 U/mg in extracts of pDEX4-17 transformants. However, the extracts showed only 5%–10% of these theoretical values. It is difficult to account for this low specific activity at this time. Assuming that more than half of the synthesized protein is imported into the peroxisomes, the low activity might be due to an incomplete folding or dimerization. The same reason might hold for the DHAS protein in the cytoplasm.

The strong overlap of peroxisomes and mitochondria in our sucrose gradients did not enable us to isolate microbodies in a pure form and led to the presence of interfering enzymatic activities in the DHAS assay. A better method for isolating peroxisomes from *S. cerevesiae* is required in order to determine the localization biochemically. We cannot exclude the possibility that the protein, although transported into the peroxisomes, is not properly assembled.

The EM data together with the fractionation data strongly indicate that at least a large part of the *DAS* product is imported into the peroxisomes. The protease resistance of the DHAS protein in the organelle fraction suggests that the protein is included in a membrane particle, whereas the immuno-labelling in the electron micrographs shows that most of the DHAS material is associated with the peroxisomal structures.

Recently Distel et al. (1987) presented evidence indicating that the *H. polymorpha* peroxisomal protein encoded by the *MOX* gene (Ledeboer et al. 1985) can also be imported into *S. cerevisiae* peroxisomes. In this case, however, no enzymatic activity could be measured, and a large part of the protein was present in cytoplasmic aggregates. The import of insect luciferase into mammalian peroxisomes represents another case of heterologous peroxisomal import (Gould et al. 1987). Moreover a target signal that allowed a cytoplasmic protein to be localized in the peroxisomal fraction could be identified at the C-terminus of the luciferase, suggesting a universal recognition system.

The study of peroxisomes in *S. cerevisiae* is greatly facilitated by the finding that the organelles increase in number and size under appropriate growth conditions (Veenhuis et al. 1987). On glucose, only a few small peroxisomes are detectable, whereas on oleic acid medium multiple larger organelles are present. By using two oppositely regulated promoters, we were able to study the effect of DHAS protein production on peroxisome morphology under both conditions. Surprisingly, the expression of the heterologous DHAS protein induced a remarkable increase in the volume of the peroxisomes. This is the best indication that DHAS protein is imported into the organelle. The increase in volume is dependent on the amount of DHAS protein produced and occurred most dramatically under conditions where normally only a small number of small peroxisomes occurs. The increase in size shows that merely the import of the protein can already lead to membrane proliferation and organelle growth.

However, no increase in number, which is normally associated with peroxisomal induction on oleate medium, was observed in DHAS-expressing cells grown

Figs. 9–12. Immunocytochemical demonstration of DHAS protein using specific antibodies against DHAS and protein A/gold. Under conditions in which DHAS is highly expressed, the microbody-matrix is densely labelled (**Fig. 10**, pIX17/oleic acid and **Fig. 11**, pDEX4-56/glucose), and labelling is present on the cytosol. Under conditions in which the synthesis of DHAS is repressed, labelling is either extremely thin (**Fig. 9**, pDEX4-56, oleic acid; *arrow*) or entirely lacking (**Fig. 12**, pIX17; glucose)

on glucose. Similar results were obtained by Distel et al. (1988) when they expressed the *MOX* gene in *H. polymorpha* during growth of the cells under peroxisome-repressing conditions. This suggests that peroxisomal biogenesis with respect to increase in both size and number underlies separate control mechanisms.

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References

Bystrykh LV, Sokolov AP, Trotsenko JA (1981) FEBS Lett 132:324–328

Carlson M, Botstein D (1982) Cell 28:145–154

Cregg JM, Barringer KJ, Hessler AY, Madden KR (1985) Mol Cell Biol 5:3376– 3385

Cross HS, Ruis H (1978) Mol Gen Genet 166:37–43

Distel B, Veenhuis M, Tabak HF (1987) EMBO J 6:3111–3116

Distel B, van der Ley I, Veenhuis M, Tabak HF (1988) J Cell Biol 107: 1669–1675

Douma AC, Veenhuis M, de Koning W, Evers M, Harder W (1985) Arch Microbiol 143:237–243

Ellis SB, Brust PF, Kontz PI, Waters AF, Harpold MM, Gingeras TR (1985) Mol Cell Biol 5:1111–1121

Erhart E, Hollenberg CP (1983) J Bacteriol 156:625–635

Goodman JM (1985) J Biol Chem 260:7108–7113

Goodman JM, Scott CW, Donahue PN, Atherton JP (1984) J Biol Chem 259:8485– 8493

Gould SJ, Keller GA, Subramani S (1987) J Cell Biol 105:2923–2931

Hollenberg CP (1979) In: Cumming DJ, Borst P, David IB, Weissman SM, Fox CF (eds), Extrachromosomal DNA, ICN-UCLA Symp on Molecular and Cellular Biology, vol 15. Academic Press, New York, pp 325–338

Hörtner HG, Ammerer G, Hartter E, Hamilton B, Rytka J, Bilinski T, Ruis H (1982) Eur J Biochem 128:179–184

Janowicz ZA, Eckart MR, Drewke C, Roggenkamp R, Hollenberg CP, Maat J, Ledeboer AM, Visser C, Verrips CT (1985) Nucleic Acids Res 13:3043–3062

Kellermann E, Seeboth PG, Hollenberg CP (1986) Nucleic Acids Res 14:8963–8977

Klebe RJ, Harris JV, Smart ZD, Douglas MG (1983) Gene 25:333–341

Laemmli UK (1970) Nature 227:680–685

Ledeboer AM, Edens L, Maat J, Visser C, Bos JW, Verrips CT, Janowicz Z, Eckart MR, Roggenkamp R, Hollenberg CP (1985) Nucleic Acids Res 13: 3063–3082

Maniatis T, Fritsch EF, Sambrooke J (1982) Molecular cloning. A laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Maxam AM, Gilbert W (1980) Methods Enzymol 65:499–560

Roa M, Blobel G (1983) Proc Natl Acad Sci USA 80:6872–6876

Roggenkamp R, Sahm H, Wagner F (1974) FEBS Lett 41:283–286

Roggenkamp R, Janowicz Z, Stanikowski B, Hollenberg CP (1984) Mol Gen Genet 194:489–493

Roggenkamp R, Hansen H, Eckart M, Janowicz Z, Hollenberg CP (1986) Mol Gen Genet 202:302–308

Schmitt HD, Ciriacy M, Zimmermann F (1983) Mol Gen Genet 192:247–252

Small GM, Szabo LG, Lazarow PB (1988) EMBO J 7:1167–1173

Tolbert NE (1974) Methods Enzymol 31:734–748

Towbin H, Staehelin T, Gordon J (1979) Proc Natl Acad Sci USA 76:4350–4354

Veenhuis M, van Dijken JP, Pilon SAF, Harder W (1978) Arch Microbiol 117: 153–163

Veenhuis M, van Dijken JP, Harder W (1983) Adv Microb Physiol 24:1–82

Veenhuis M, Mateblowski M, Kunau WH, Harder W (1987) Yeast 3:77–84

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